

Review

The ubiquitous mountain hare mitochondria: multiple introgressive hybridization in hares, genus *Lepus*

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Climatic oscillations during the glaciations forced dramatic changes in species distributions, such that some presently temperate regions were alternately occupied by temperate and arctic species. These species could have met and hybridized during climatic transitions. This phenomenon happened for three hare species present in Iberia (*Lepus granatensis*, *Lepus europaeus* and *Lepus castroviejoi*), which display high frequencies of mitochondrial DNA (mtDNA) from *Lepus timidus*, an arctic/boreal species presently extinct in Iberia. Here, we extend our previous geographical survey to determine whether the distribution of this mtDNA lineage extends beyond the northern half of the Iberian Peninsula, where it is found at high frequencies. We also review the taxonomy, distribution and molecular phylogeny of the genus *Lepus*. The phylogenetic inference reveals the presence of *L. timidus*-like mtDNA in several other hare species in Asia and North America, suggesting that the mitochondrial introgression observed in Iberia might be generalized. Comparison with the available nuclear gene phylogenies suggests that introgression could have happened repeatedly, possibly during different climatic transitions. We discuss demographic and adaptive scenarios that could account for the repetition in time and space of this spectacular phenomenon and suggest ways to improve our understanding of its determinants and consequences. Such high levels of introgressive hybridization should discourage attempts to revise hare taxonomy based solely on mtDNA.

Keywords: hybridization; mitochondrial DNA introgression; hares; Iberian Peninsula; selection

1. INTRODUCTION

Classifying living organisms into species and reconstructing phylogenetic relationships remain a major endeavour of naturalists. However, since populations descend from each other, speciation is continuous by essence and attempting to classify the outcomes of this process into discrete categories often comes to squaring the circle. Obvious obstacles to the rough application of any automatic classification criteria include shared ancestral polymorphism, character convergence and exchange via secondary hybridization. The advent of molecular genetics has theoretically given us the power to assess the influence of these three confounding

factors, to appreciate the complexity of the speciation process and to understand the historical and genomic differentiation processes among populations before deciding how the notions of species and phylogenies apply to them. The view that the history of populations and species could be faithfully depicted by the history of a single gene or portion of the genome has been rewarding when studying sufficiently distantly related organisms. However, it can be highly misleading when closely related taxa are compared, precisely the level of relatedness relevant to understanding the speciation process.

Among the phenomena that challenge the classification of organisms, hybridization and introgression are relatively easy to characterize and have attracted much attention. These events have long been considered fundamental in plant evolution, but the importance of their role in animal evolution was only recently recognized, as more cases of hybridization came to light (see Dowling & Secor 1997). As many as 10% of animal species (primarily evolutionarily 'young' species) may be influenced by hybridization and

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introgression (Mallet 2005). Cases of introgression reported in the literature frequently concern mitochondrial DNA (mtDNA) with variable levels of mitochondrial replacement (see, e.g. Avise 2004; Ballard & Whitlock 2004). The mtDNA introgression is most often reported in situations of current contact and hybridization between species, such as in *Drosophila* (Powell 1983), *Clethrionomys* (Tegelström 1987), *Thomomys* (Ruedi *et al.* 1997), *Cervus* (Goodman *et al.* 1999) and *Myotis* (Berthier *et al.* 2006). However, we know that the establishment and disruption of contacts between species mostly result from shifting ranges in response to climatic changes. In the Northern Hemisphere, temperate species tended to retract to the south during the glacial periods (Hewitt 1996). In Europe, the southern peninsulas (Iberia, Italy and the Balkans) were the most important refugial areas for these species and are considered important repositories of biodiversity (Taberlet *et al.* 1998). While periods of retraction can give rise to differentiation between isolated refugial populations, periods of expansion can lead to the establishment of secondary contacts and, possibly, to admixture or partial introgression. Although much less studied, arctic species are expected to have a different reaction to the cooling of the climate, and they tend to maintain large areas of distribution south of the ice rim (Hewitt 2001). During climate warming while temperate species expanded from the southern refugia (Hewitt 1996; Cruzan & Templeton 2000), arctic species moved to the North accompanying the retreat of the glaciers. Hence, the arctic and temperate fauna must have repeatedly replaced each other in large geographical regions, with the possibilities of transient contacts and hybridization between closely related arctic and temperate species. We have found evidence for this phenomenon in the Iberian Peninsula where the three extant temperate hare species harbour high frequencies of mtDNA from an arctic species, *Lepus timidus* (Alves *et al.* 2003; Melo-Ferreira *et al.* 2005, 2007). Here we extend our previous geographical survey of this phenomenon in Iberia, and also review the available molecular data on other hare species. On the basis of this preliminary evidence, we argue that the phenomenon is not limited to Iberia but could have happened all over the former range of the arctic species and with several other hare species from different regions.

2. THE GENUS *LEPUS*: ORIGIN, TAXONOMY AND CURRENT DISTRIBUTION

Taxonomy of members of the genus *Lepus*, a polytypic genus in the Lagomorph family Leporidae, remains somewhat confusing mostly due to a strong overlap of morphological characters, an invariable karyotype across species ($2n=48$) and the lack of extensive molecular studies (see, e.g. Flux & Angermann (1990), Robinson & Matthee (2005) and references therein). A recent mtDNA and nuclear DNA (nDNA) analysis suggested that the genus *Lepus* most probably originated in North America *ca* 12 Myr ago and then dispersed across the other continents via an ancestral lineage that crossed the Bering Strait to Asia *ca* 7–5 Myr ago (Matthee *et al.* 2004). Although mtDNA sequence studies at finer time scales have contributed to clarify

some phylogenetic uncertainties (Halaných *et al.* 1999; Pierpaoli *et al.* 1999; Alves *et al.* 2003; Wu *et al.* 2005; Ben Slimen *et al.* 2007, 2008), they have also been a source of confusion when revealing unexpected genetic similarities among taxa formerly considered divergent. For example, Halaných *et al.* (1999) analysed 11 recognized species of *Lepus* predominantly from North America and showed that the North American *Lepus* group is not monophyletic, which suggests a reflux of species from Asia. In addition, other studies have clearly demonstrated that several other *Lepus* species are not monophyletic based on mtDNA analyses (e.g. Alves *et al.* 2003; Wu *et al.* 2005).

The genus *Lepus* is cosmopolitan, with representatives on all continents and in a great variety of habitats. While most hare species have rather limited distributional ranges (e.g. *Lepus corsicanus* in Italy, *Lepus castroviejo* in Spain or *Lepus insularis* in Mexico), some cover vast areas, sometimes across different continents and quite dissimilar habitats, such as *Lepus capensis* and *L. timidus* (Alves & Hackländer 2008). The palaeontological records show that the distribution of some species changed greatly during climate cycles, as is the case of the mountain hare, *L. timidus*. Although this species is clearly arctic/boreal and adapted to cold conditions, it seems capable of inhabiting a wide range of biota, from permafrost to rich agricultural lands. Presently, it has a wide, almost circumpolar, distribution in the Northern Hemisphere, ranging from the British Isles to the Russian Far East, with some isolated populations in the Alps, Poland and Japan (Angerbjörn & Flux 1995; figure 1). However, palaeontological records of this species witness a very different past distribution, with Upper Pleistocene fossil evidence demonstrating its historical presence, e.g. in southern France (Lopez-Martinez 1980) or in the Iberian Peninsula (Altuna 1970). Recent studies using mtDNA and microsatellites (Hamill *et al.* 2006), or only mtDNA but with a more extensive sampling (Melo-Ferreira *et al.* 2007), suggested that *L. timidus* most probably maintained large populations during the glacial periods, presumably south of the ice sheet, from where it must have later been displaced by temperate species.

3. MASSIVE MITOCHONDRIAL DNA INTROGRESSION FROM *L. TIMIDUS* INTO THREE SPECIES IN THE IBERIAN PENINSULA

The current taxonomic view accepts the presence of three species of hares in the Iberian Peninsula: *Lepus granatensis*; *Lepus europaeus*; and *L. castroviejo* (figure 2). Each of these species is characterized by a mitochondrial DNA lineage of its own, but in Iberia all three species also share a fourth lineage, hereafter labelled 'foreign', the representatives of which are more related to each other than they are to any of the three species-specific lineages (Alves *et al.* 2003; Melo-Ferreira *et al.* 2005). The average divergence of the foreign lineage to *L. granatensis* and *L. europaeus* is approximately 10%, a typical value for interspecific differences in mammals (Johns & Avise 1998), while the divergence to *L. castroviejo* is approximately 3% (Alves *et al.* 2003). This foreign lineage is clearly related to mtDNA of *L. timidus*, which suggests that its presence in the Iberian species resulted from secondary



Figure 1. Current species ranges of *L. europaeus* and *L. timidus* in Eurasia according to Flux & Angermann (1990) and Mitchell-Jones *et al.* (1999). The dashed box depicts the Iberian Peninsula. (See figure 2 for the ranges of hare species in this region.)

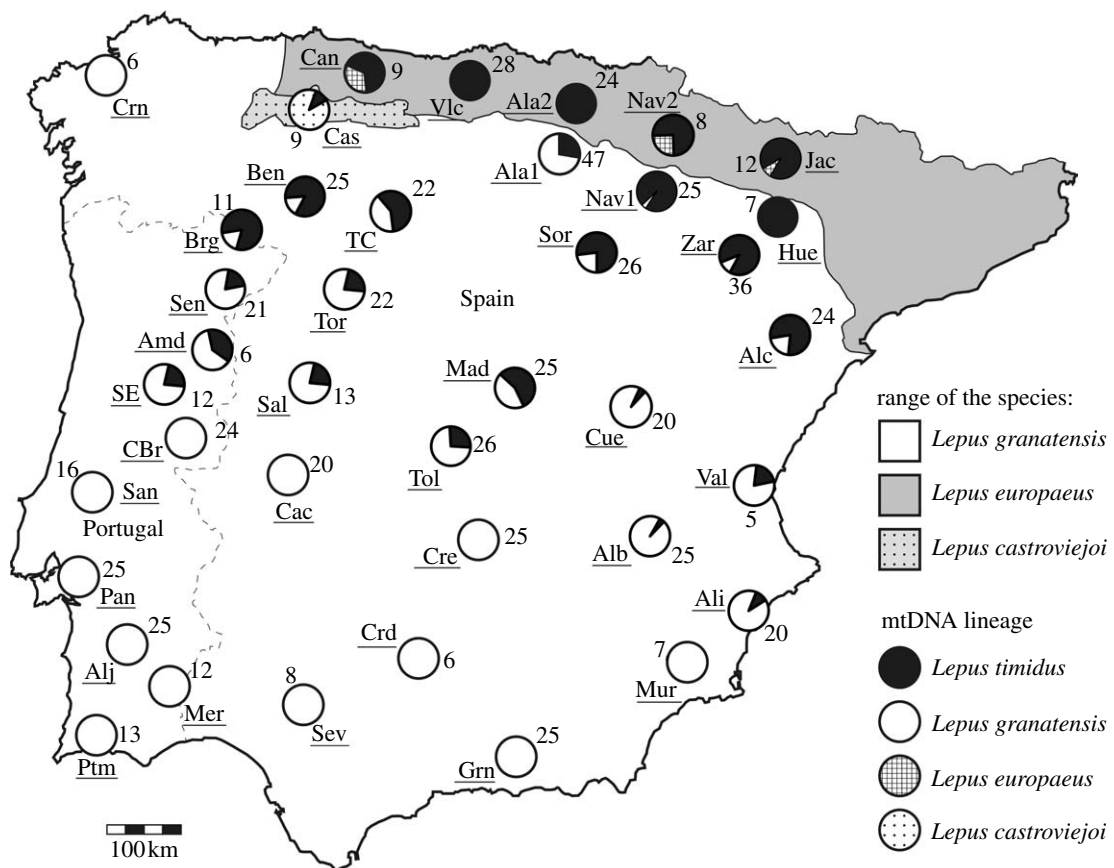


Figure 2. Ranges of the species of hares in the Iberian Peninsula and geographical distribution and frequencies of the four mitochondrial lineages observed in 39 populations (updated from Melo-Ferreira *et al.* 2005): 33 from *L. granatensis* (Ala1, Álava; Alb, Albacete; Alc, Alcañiz; Ali, Alicante; Alj, Aljustrel; Amd, Almeida; Ben, Benavente; Brg, Bragança; CBr, Castelo Branco; Cac, Cáceres; Crd, Córdoba; Crn, La Coruña; Cre, Ciudad Real; Cue, Cuenca; Grn, Granada; Hue, Huesca; Mad, Madrid; Mer, Mértola; Mur, Murcia; Nav1, Navarra; Pan, Páncas; Ptm, Portimão; Sal, Salamanca; SE, Serra da Estrela; San, Santarém; Sen, Sendim; Sev, Sevilla; Sor, Soria; TC, Tierra de Campos; Tol, Toledo; Tor, Tordesillas; Val, Valência; Zar, Zaragoza); 5 from *L. europaeus* (Ala2, Álava; Can, Cantabria; Jac, Jaca; Nav2, Navarra; Vlc, Villarcayo) and 1 from *L. castroviejoi* (Cas, Cantabria). Sample sizes and population codes are shown next to the pie charts.

introgression rather than from the persistence of ancestral polymorphism in the Iberian species. This conclusion is reinforced by phylogenies inferred from nuclear genes that clearly identify each of the Iberian species as a monophyletic clade (Alves *et al.* 2003, 2008), although the nuclear genome is known to require more time to reach reciprocal monophyly after speciation than the mtDNA. The mtDNA introgression was found to be extensive with the *L. timidus* haplotypes being predominant in the Iberian range of *L. europaeus* as well as in the northern range of *L. granatensis*, disappearing further south (Melo-Ferreira *et al.* 2005). We updated this survey, reaching a total of 720 specimens (630 *L. granatensis*, 81 *L. europaeus* and 9 *L. castroviejoi*) from 39 locations across the Iberian Peninsula, including previously unsampled populations (figure 2). These new data reveal the presence of *L. timidus* haplotypes as far south as Serra da Estrela in Portugal and Alicante in Spain.

The palaeontological data attest to the historical presence of *L. timidus* in the Iberian Peninsula, and the genetic data demonstrate that it hybridized with the Iberian species and left traces before going extinct from this region. The amplitude of this molecular trace is spectacular in its geographical and taxonomic extent (figures 2 and 3). *L. timidus* could have occupied a significant part of Iberia during the last glaciation peak, and its extinction was presumably preceded by competitive replacement by the more adapted temperate Iberian species as it became warmer during deglaciation. Currat & Excoffier (2004) have simulated such scenarios of competitive replacement and concluded that the traces of hybridization events at the contact zone, even if rare, could be amplified by the demographic expansion dynamics of the invading species. This eventually leads to high frequencies of alleles from the outcompeted species at the front of expansion of the invading species. The phenomenon could even be amplified as the invading species continues to expand, propagating the foreign alleles at even higher frequencies into its newly conquered territories. Both characteristics are clearly observed in *L. granatensis* (figure 2; Melo-Ferreira *et al.* 2005). Furthermore, the coalescence patterns of *L. timidus* haplotypes found in *L. granatensis* suggest a sudden demographic expansion, estimated to have occurred roughly at the end of the glacial period (Melo-Ferreira *et al.* 2007), which is also coherent with the proposed scenario. The *L. timidus* mtDNA introgression into Iberian *L. europaeus* is more extensive than that in *L. granatensis*, having led to a quasi-replacement of the original mtDNA (figure 3). It is however not known whether *L. timidus* existed in Iberia at the time *L. europaeus*, which contrary to *L. granatensis* is not endemic to Iberia, entered the Peninsula. Since it is now known that *L. europaeus* and *L. granatensis* hybridize in the north of the Iberian Peninsula (Freitas 2006), they could have passed the mtDNA of *L. timidus* origin to each other. However, current gene flow between the species appears limited and could not have led to the quasi-fixation of *timidus* mtDNA in Iberian *europaeus* unless introgression was favoured by strong selection (Freitas 2006; Melo-Ferreira *et al.* 2007).

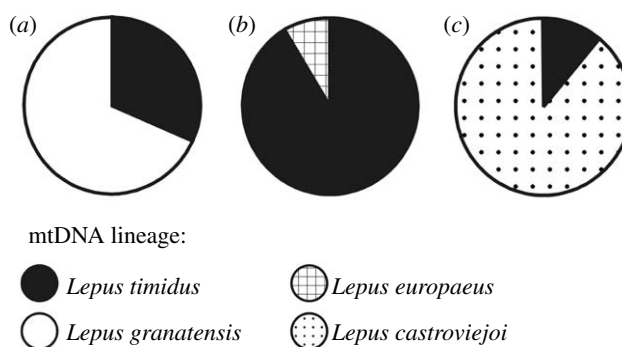


Figure 3. The proportion of the mountain hare mitochondrial lineage within the three hare species from the Iberian Peninsula: (a) *L. granatensis* with 31.7% *L. timidus* mtDNA, (b) *L. europaeus* with 92.3% *L. timidus* mtDNA and (c) *L. castroviejoi* with 11.1% *L. timidus* mtDNA.

The lack of records on the history of *L. europaeus* in Iberia invites caution in applying to this species the same scenario of competitive replacement of *L. timidus* as described for *L. granatensis*, although some observations suggest that the phenomenon may be occurring in areas of present contact between *L. timidus* and *L. europaeus*. mtDNA introgression from *L. timidus* into populations of *L. europaeus* has been known for a decade in Sweden (Thulin *et al.* 1997; Thulin & Tegelström 2001, 2002). According to these studies, native *L. timidus* hybridize with introduced *L. europaeus*, a species that is progressively replacing *L. timidus*. In artificial crosses between these species, mating occurs only in the direction required to account for the observed introgression, i.e. *L. timidus* females with *L. europaeus* males (Gustavsson & Sundt 1965). Introgression of *L. timidus* was also recently reported in *L. europaeus* from Denmark, but could result from the introduction of already introgressed hares from Scandinavia (Fredsted *et al.* 2006). What is presently observed in Scandinavia might be highly similar to what happened in Iberia 10 000 years ago: the arctic species tends to be overtaken by the temperate one on the southern border of its distribution, leaving mtDNA traces as it retreats.

4. APPARENT ABSENCE OF NUCLEAR INTROGRESSION AND UNIDIRECTIONAL mtDNA TRANSMISSION

Given the high frequency of mtDNA of *L. timidus* origin in Iberia, one could expect some level of parallel introgression of nuclear genes. The analysis of 14 autosomal protein loci in *L. granatensis* populations did not show any significant differentiation among populations where the *L. timidus* mtDNA is present and those where it is not ($F_{ST}=0.01$; table 1; Alves 2002). This apparent lack of nuclear introgression is also supported by microsatellite analyses (Estonba *et al.* 2006; Freitas 2006). Further assessment is clearly needed; but from this preliminary data it appears at least unlikely that nuclear introgression could be of the same magnitude as that of mtDNA.

Contrasting genetic patterns between nuclear and mitochondrial markers are usually explained either by higher genetic drift of mtDNA (due to reduced effective size of mtDNA when compared with nDNA) or by

Table 1. Weir & Cockerham's estimates of F -statistics across protein loci in *L. granatensis* for group A (absence *L. timidus* mtDNA), group B (presence of *L. timidus* mtDNA) and between groups (significance difference from zero: * $p < 0.05$; ** $p < 0.01$).

	F_{IS}	F_{IT}	F_{ST}
group A	0.00	0.08**	0.08**
group B	−0.01	0.00	0.01
between groups	0.05*	0.06**	0.01

male-biased dispersal (Palumbi & Baker 1994). As commonly observed among mammals, male hares are typically more mobile than females (Bray 1998). Considering the replacement with hybridization scenario in Iberia, if *L. granatensis* males were the first to invade the *L. timidus* range, the cross of *granatensis* males with *timidus* females would be favoured, resulting in unidirectional introgression. Similarly, unidirectional gene flow from *L. timidus* into *L. europaeus* was observed in Sweden (Thulin et al. 1997) and Denmark (Fredsted et al. 2006), but in Russia (Thulin et al. 2006) and the Alps (Suchentrunk et al. 2005) it also occurs in the reverse direction. Thus, while an asymmetry seems to exist, it does not seem to be consistent in all known cases of current hybridization events. However, the recurrent cross of hybrid females with males of the invading species, coupled with male hybrid sterility or inviability (Haldane's rule), would suffice to completely dilute the nDNA of one species in a few generations (see Roca et al. 2005).

5. CIRCUMPOLAR MITOCHONDRIAL DNA INTROGRESSION?

Looking at the present distribution of *L. timidus* (figure 1) and considering that it was able to maintain a large and continuous area of distribution during the glacial periods (Melo-Ferreira et al. 2007), it seems probable that *L. timidus* was in contact with several other species during the glacial/post-glacial range shifts. Therefore, we reanalysed the available data on the molecular phylogeny of hares. Some recent studies have tried to assess the phylogenetic relationships of hare species using mtDNA alone (Waltari & Cook 2005; Wu et al. 2005; but see Alves et al. 2006). For example, when analysing Chinese hare relationships, Wu et al. (2005) concluded that *Lepus capensis*, a species with a broad distribution from Africa to China, does not occur in China since its mtDNA is similar to that of *L. timidus*. Additionally, in the phylogeny presented by these authors, some species were clearly paraphyletic, with some sequences in the *L. timidus* clade and others in another distant clade. Previously, Yu (2004) had proposed that the Chinese hares most probably derived from *L. timidus*. However, in China as in Iberia, there is evidence for the presence of *L. timidus* during the glacial periods in places where it is currently absent (Zhang 2002).

Another example of possible erroneous taxonomic inferences due to phylogenetic assessments based only on mtDNA is found in the arctic hare complex. This complex includes *L. timidus* from Eurasia, which is

replaced by *Lepus othus* in Beringia and by *Lepus arcticus* in North America and northern Greenland (Flux & Angermann 1990). Waltari & Cook (2005) found in their mtDNA phylogeny of the arctic hares that the *L. timidus* mtDNA clade also included haplotypes from *L. othus* and *L. arcticus*. They argued that the monophyly of both *L. arcticus* and *L. othus* sustain their species status but disregarded that accepting this view made *L. timidus* paraphyletic. This pattern might result from incomplete lineage sorting of mtDNA in these closely related taxa, but hybridization is certainly an alternative that should not be overlooked. Of course, only extensive characterizations of these species and their nuclear genome would give reliable answers to these questions, but there are many other cases where mtDNA introgression from *L. timidus* is a plausible hypothesis to explain the mtDNA phylogeny. In figure 4 we present a phylogeny of 19 species of hares using a set of published cytochrome *b* sequences. A clade containing all *L. timidus* also contains 11 other species (not only *L. granatensis*, *L. europaeus*, *L. castroviejoi*, *L. arcticus*, *L. othus* and *L. capensis*, which we already discussed, but also *L. corsicanus*, *Lepus mandshuricus*, *Lepus townsendii*, *Lepus oiostolus* and *Lepus tolai*), and some of these species are also found elsewhere on the tree (*L. granatensis*, *L. europaeus*, *L. capensis*, *L. oiostolus* and *L. mandshuricus*). Interestingly, *L. mandshuricus* from Manchuria, *L. townsendii* from North America, *L. oiostolus* from Tibet and *L. tolai* from Russia, Mongolia and China have geographical distributions that seem compatible with present and/or past contacts with *L. timidus*.

In the mtDNA phylogeny of figure 4, *L. castroviejoi* appears to split into two groups of haplotypes: one falls with the majority of *L. timidus* specimens and with other Iberian haplotypes of *L. timidus*, while the second group falls into a sister clade of *L. corsicanus*. In discussing the situation in Iberia above, we considered that the latter group of haplotypes might represent a mtDNA lineage specific to this species while the former represented introgression from *L. timidus*. This would imply that *L. castroviejoi* is closely related to *L. timidus*. However, this is contradicted by nuclear gene phylogenies showing that *L. timidus* is roughly equidistantly related to *L. castroviejoi*, *L. granatensis* and *L. europaeus* (Alves et al. 2003, 2008). Therefore, we suggest that the true original *L. castroviejoi* mtDNA has not been sampled, but that instead the two lineages of mtDNA found in this species are of *L. timidus* origin and witness two introgression events of different ages. Whether the original mtDNA lineage still exists in *L. castroviejoi* will have to be assessed by further sampling.

6. SELECTIVE ADVANTAGE OF MOUNTAIN HARE mtDNA

Almost all known cases of hybridization in the genus *Lepus* involve *L. timidus* and mtDNA introgression from this species seems to be a common phenomenon. Since introgressive hybridization occurred frequently and multiple times independently in different species, we assume that deterministic forces have shaped this process. The scenario of competitive replacement during climate warming that we put forward to explain the genetic differentiation among Iberian hare

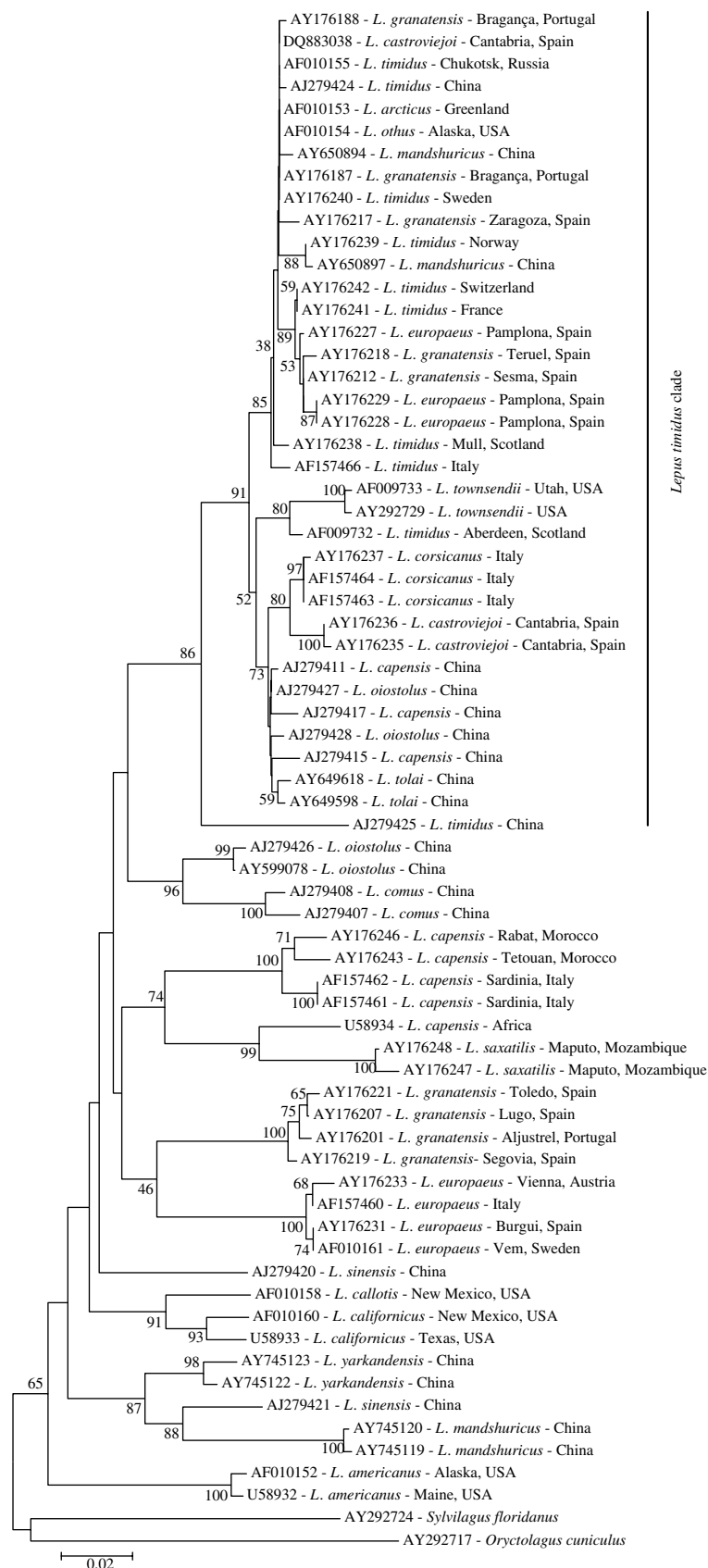


Figure 4. Neighbour-joining tree of cytochrome *b* sequences (552 bp) of 19 species of hares, computed using the Kimura two-parameter model of evolution (Kimura 1980) and 1000 bootstrap replicates, using the program MEGA v. 3.1 (Kumar et al. 2004). Sequences from the European rabbit (*Oryctolagus cuniculus*) and cottontail (*Sylvilagus floridanus*) were used as outgroups. All sequences were downloaded from GenBank (accession numbers are depicted on the tips of the tree). Species names and specimen locations are also indicated.

populations might be used to elucidate similar patterns found in other geographical regions. But the reverse phenomenon could also have happened in periods of

climate cooling, with replacement of the temperate species by the one from the Arctic. However, the data do not provide any evidence that mtDNA introgression

has occurred from temperate to arctic species. Perhaps more refined palaeoecological studies could provide an explanation to this asymmetric pattern since changes in the environment need not have occurred at the same pace during warming and cooling periods. The asymmetric pattern could also result from behavioural asymmetry if female *L. timidus* were particularly tolerant to allospecific matings when compared with females of other species. Finally, it might be that mtDNA introgression of *L. timidus* was facilitated by natural selection if cytonuclear recombinants gained an advantage under certain conditions. Evidence is accumulating that mtDNA is subject to selection pressures of various sorts (reviewed by e.g. Rand 2001; Ballard & Whitlock 2004). In particular, because the mitochondrial metabolism is involved in heat production, mtDNA could participate in adaptation to cold (Mishmar et al. 2003; Ruiz-Pesini et al. 2004; Fontanillas et al. 2005). It could thus be that the spread and persistence of mtDNA of *L. timidus* in the northern ranges of the temperate species, in which it was introduced, were facilitated by conferring some form of resistance to low temperatures. The coalescent pattern that we observed in the lineages of *L. timidus* from Iberian hares (Melo-Ferreira et al. 2007) is compatible with either positive selection or demographic expansion. Differentiating between the two effects would require comparison with other unlinked (therefore nuclear) loci (e.g. Galtier et al. 2000). However, nuclear introgression appears to be scarce (see above, and Melo-Ferreira et al. 2008, unpublished data), and the comparison between these two types of loci with different modes of transmission would not be very powerful, especially in a context where demographic expansion is anyway likely to have occurred. We rather suggest two alternative approaches to answer this question. One would be to assess the level of introgression of nuclear candidate genes that participate in the metabolic chain of heat production in the mitochondria. If adaptation to low temperatures plays a role, then we should observe a differential pattern of introgression comparing functional and neutral genes. The second approach would be to compare the coalescent patterns of indigenous and introduced mtDNA lineages among the populations that possess both lineages. If mtDNA is neutral, both lineages should reflect the same demographic processes since they evolved in the same populations, at least since the initial contact between the species. This is an interesting but difficult question in population genetics. The ultimate answer to the question of adaptation would of course come from physiological studies on hares with different mtDNA haplotypes.

7. CONCLUSION

The phenomenon of ancient mitochondrial introgression observed in hares from Iberia can be satisfactorily explained by hybridization and competitive replacement during climate warming. However, there are still uncertainties about the exact circumstances under which it occurred, about the role of selection and the impact on the rest of the genome. The preliminary data presented here suggest that similar phenomena have

occurred in several other regions between *L. timidus* and other hare species, offering potential replicates of the same phenomenon, and thus a valuable context for comparative evolutionary studies.

All new data included in this paper have been carried out following the Portuguese and Spanish ethics rules.

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